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Instability of Expression of Lipooligosaccharides and Their Epitopes in *Neisseria gonorrhoeae*

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We assessed variation in the expression of lipooligosaccharide (LOS) components and their epitopes within populations of a strain of *Neisseria gonorrhoeae* by using the monoclonal antibodies (MAbs) O6B4 and 3F11 and immunoenzymatic, immuno-colloidal gold electron microscopic, and sodium dodecyl sulfate-polyacrylamide gel electrophoretic procedures. Wild-type organisms varied in binding of both MAbs. We used the intensity of immunoenzymatic colony blot color to distinguish four binding variants for each MAb: red (R), pink (P), and colorless (nonreactive [N]) and an N back to R (N-R) revertant. R to P to R and R to N to R variation occurred at frequencies of 0.2% and 0.02%, respectively. The electrophoretic LOS profiles and MAb immunoblot patterns of the R, P, and N-R variants were the same as those of the wild type. LOSs of the N variants, in contrast, were of lower M_r , bound neither 3F11 nor O6B4 MAb, and contained as their major component the 3.6-kilodalton LOS that bears the L8 LOS epitope of *N. meningitidis*. Results of immunoelectron microscopic studies were consistent with LOS binding patterns. Large numbers of colloidal gold particles were deposited about both R and P variants, distally from R organisms, but proximally from P organisms. N variant organisms, like their LOS, bound neither of the MAbs. N-R variant organisms were like the wild type in that they showed much variation in the amounts of MAb they bound.

The principal outer membrane glycolipids of *Neisseria gonorrhoeae* are a series of lipooligosaccharides (LOSs) of M_r between 3,200 and 7,200 (16). Each gonococcal strain makes between one and six antigenically and physically distinct LOS molecules (7, 16; J. M. Griffiss, H. Schneider, R. E. Mandrell, R. Yamasaki, G. A. Jarvis, B. Gibson, R. Hamadeh, and M. A. Apicella, Rev. Infect. Dis., in press). Whereas the total number of different LOSs that can be made by *N. gonorrhoeae* is extensive (7), the LOS repertoire of any strain is limited and is a stable population attribute of that strain. This is indicated by the fact that the number and relative quantity of each LOS produced by different populations of the same strain vary little (3, 17).

The stability with which the molecular heterogeneity of the LOS repertoire of a strain is expressed by populations of that strain implies that LOS production is regulated at the single-cell level (3). Such regulation could be reflected in two different patterns: either by each cell's making a regulated number of each LOS of the repertoire, or by each cell's making a predominant LOS of that repertoire and the relative number of individual cells of each LOS phenotype being regulated.

Our recent studies suggest that the LOS phenotype is unstable (3). Individual cells within a population bound different amounts of each of two monoclonal antibodies (MAbs), 3F11 and O6B4 (3). Most cells bound both antibodies to some degree, but some cells bound only one or the other. Furthermore, the distribution of epitope densities among a population of cells, assessed by fluorescence-activated sorting of MAb-labeled cells, varied with growth phase (3). This is consistent with previous reports that the LOS repertoire may be influenced by growth conditions (9, 11, 12) and supports the conclusion that each cell is capable of making the full LOS repertoire of the strain. Regulation of

the LOS phenotype of a gonococcal strain by growth conditions has obvious biological implications. The LOS repertoire of a strain that is colonizing a human mucous membrane might be quite different from that of the same strain grown on artificial medium.

We designed the present studies to define further the instability of LOS expression by gonococcal cells. The results indicate that spontaneous variation in the LOS repertoire of cells within a strain's population occurs in the absence of selective pressure. This variation is manifested as qualitative changes in LOS molecules and their epitopes. The sum of these spontaneous variations accounts for the heterogeneity of the population's LOS repertoire.

MATERIALS AND METHODS

Bacteria and culture conditions. Wild-type (WT) *N. gonorrhoeae* 4505 has been characterized (1). We grew it and its variants at 37°C in humidified candle extinction jars on GC medium (Disco Laboratories, Detroit, Mich.) supplemented with 3 g of glucose, 8.3 mg of Fe(NO₃)₃ · 9H₂O, 1 g of L-glutamine, 1 ml of 0.2% carboxylase, and 40 mg of L-cysteine per liter (GCDC).

Antibodies. Development and production of the murine MAbs O6B4 and 3F11 have been described (2); they are both of the immunoglobulin M (IgM) isotype. They recognize different epitopes present on the same LOS or on closely migrating LOS components (7). Production of the murine MAb 2-1-L8, an IgG3 which recognizes the L8 epitope borne on a 3.6-kilodalton (kDa) LOS made by some *Neisseria meningitidis* and *N. gonorrhoeae* strains, has also been described (15, 22). We obtained goat anti-murine secondary antibodies from Kirkegaard and Perry, Gaithersburg, Md.

IEZ detection of LOS variants. We identified LOS variants on blots of agar-grown master cultures of well-separated 4505 WT colonies made directly onto nitrocellulose filters (9-cm diameter, 0.45-μm pore size; Millipore Corp., Bedford,

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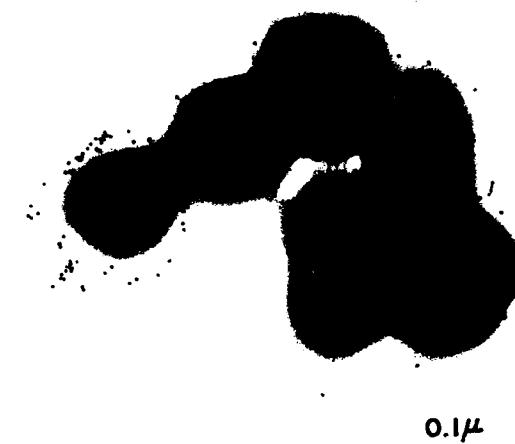


FIG. 1. IEM demonstrating nonuniform binding of MAb O6B4 onto the surfaces of WT 4505 organisms and, therefore, variable expression of the LOS epitope that it defines. Bar, 0.1 μ m.

Mass.) by an immunoenzymatic (IEZ) procedure (19). We reincubated the master cultures as described above for an additional day; in the interim we processed the colony blots for IEZ. The blots were air dried and then incubated for 30 min in 2% casein-phosphate-buffered saline, pH 7.6 (C-PBS), to block nonspecific protein-binding sites. We reacted the blots with either MAb O6B4 or 3F11 diluted in C-PBS for 2 or 18 h, respectively, and then washed them thrice with PBS. We then incubated the blots with an alkaline phosphatase-conjugated goat anti-murine immunoglobulin secondary antibody diluted in C-PBS for 1.5 h and washed them twice as described above and once with 0.05 M Tris buffer, pH 8.3. We detected immunoreactive colonies as red blots by immersing the filters in 15 ml of developing reagent (0.1% naphthol AS MX phosphoric acid [disodium salt] and 0.2% fast red TR salt) (both from Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.05 M Tris buffer, pH 8.3. We identified variant colonies on the reincubated master cultures after comparing them with their color-developed colony blots. The variants were passed thrice and then retested for stability. We suspended single variant colonies in Mueller-Hinton broth (Difco) and plated appropriate dilutions on GCDC medium. We scored for color revertants among the blots of 1,500 and 2,000 colonies by IEZ and calculated rates as the percentage of revertants among the total number of colonies tested.

SDS-PAGE and immunoblotting of LOS components. We obtained LOS by treating lysed whole cells with proteinase K (Boehringer-Mannheim, San Francisco, Calif.) by the procedure of Hitchcock and Brown (6). We separated their LOSs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) and visualized them by silver staining (20). We loaded replica gels with amounts of proteinase K lysates that contained approximately 0.7 μ g of LOS, as determined by comparison with purified LOS standards (16). We stained one gel with silver and electroblotted its replica to nitrocellulose (16). We reacted electroblotted LOS sequentially, first with either 3F11 or O6B4 MAbs as described for IEZ of colony blots above, second with 2-1-L8 MAb as described previously (16), and lastly with the converse of the first MAb. For the second and third

MAbs, horseradish peroxidase-conjugated secondary antibodies were used, and blue color developed in a substrate mixture consisting of equal parts of 4-chloro-1-naphthol (0.3 mg/ml) (Sigma) and 0.001% hydrogen peroxide, each in 0.05 M Tris buffer, pH 7.6. We photographed the blot after each successive MAb treatment. Because O6B4 and 3F11 (both IgM) bind to the same LOS component, we performed experiments to demonstrate that under the conditions used here the initial MAb was saturated by the first conjugate. In these experiments LOS blots were reacted first with MAb and then successively with alkaline phosphatase- and horseradish peroxidase-conjugated secondary antibodies; we could not detect any additional binding of the second conjugate.

IEM. For immuno-colloidal gold electron microscopy (IEM), we used colloidal gold (5- μ m particles)-conjugated goat anti-murine IgM (Janssen Biotechnology, Paramus, N.J.) as the secondary antibody to visualize binding of either O6B4 or 3F11 MAb to the surfaces of intact organisms (3). Organisms were fixed in 3% Formalin in PBS and applied to Formvar-coated nickel grids. We reacted the grids with either O6B4 or 3F11 and then with colloidal gold-labeled secondary antibody (3). The finished grids were examined in a Siemens-Elmskop transmission electron microscope.

RESULTS

Figure 1 shows a representative IEM of O6B4-treated WT 4505 cells. The population of cells was clearly heterogeneous in expressing the epitope identified by MAb O6B4. There was considerable variation in both the amount and pattern of MAb binding to each cell. Some cells bound MAb circumferentially but at some distance from the cell surface; other cells bound MAb close to the surface. Many cells bound little or no MAb. We found the same variation in the binding of MAb 3F11 by WT 4505 cells.

We used IEZ analysis of colonies to resolve the heterogeneity of MAb binding patterns. The IEZ blots of most colonies were deep red (R), but we found occasional pink (P) blots or blots with red and pink sectors (Fig. 2A). R and P variants derived from a sectored colony were stable after three single-colony passes. However, either variant gave rise to a third, MAb-nonreactive variant (N), which we detected as an uncolored sector in an MAb-reactive colony blot (Fig. 2B). On passage, N variants gave rise to a fourth



FIG. 2. IEZ identification of 3F11 MAb-reactive colony blot variants of WT 4505. (A) R- and P-sectored colony blot (arrow). (B) R colony blot (arrow) which contains a small 3F11-nonreactive (N) sector.

variant, designated N-R, which appeared to be a reversion to the original R variant. These variations occurred at the following frequencies with both MAbs 3F11 and O6B4: R to P, P to R, and R to N, 5×10^{-2} ; N to R, 5×10^{-3} . Variation from N to P was not detected.

We sought an explanation for the differences in IEZ-derived color intensity of the variant colony blots in the epitope content or the electrophoretic mobility (physical size) of their LOSs. The SDS-PAGE profiles and MAb reactivities of LOS from WT 4505 and its O6B4- and 3F11-selected variants are shown in Fig. 3. The WT and R, P, and N-R variants each made the same five LOS components (Fig. 3A), with the third, or middle one in electrophoretic mobility, in greatest abundance. Although the relative abundance of LOS components varied somewhat among the variants, there was little overall difference. The LOSs of the two N variants were different from those of the WT and the other variants (Fig. 3A): they contained two small LOS components that were in greatest abundance and two larger components that were barely detectable by the silver stain.

Both 3F11 and O6B4 bound the dominant third LOS component of the WT, R, P, and N-R variants, as well as the largest, least mobile component (Fig. 3B and D). The smallest, most abundant (fourth) N variant LOS bound MAb

2-1-L8 (Fig. 3C), indicating that it is the 3.6-kDa LOS that bears the L8 epitope (15). A 3.6-kDa LOS was also made by the other variants (Fig. 3A) in quantities sufficient to be visualized by silver stain but insufficient to be detected by MAb 2-1-L8.

Neither SDS-PAGE profiles nor immunoblot analyses of R and P variant LOSs accounted for the differences in IEZ-derived colony blot color. We therefore examined organisms of each of the variants by IEM to learn whether they differed in the way they bound the MAbs to their surfaces. Large amounts of immuno-colloidal gold particles were deposited on both R organisms (Fig. 4A) and P organisms (Fig. 4B). However, the particles were deposited consistently in a distal, circumferential pattern about R variant organisms but close to the surface of P variant organisms. Unlike WT 4505 organisms, which showed great variation in the amounts of MAb bound to their surfaces (Fig. 1), R and P variant organisms all bound large amounts of MAb, albeit in spatially different patterns. As expected, N variants did not bind the MAb (Fig. 4C). The latter served as a negative control for nonspecific binding of either the MAb or the secondary antibody. N-R revertant organisms (not shown) resembled WT organisms in that they bound various amounts of MAb.

DISCUSSION

The data confirm that a population of gonococcal cells of a single strain consists of several phenotypically different variants, each of which makes a predominant LOS, and which may interconvert at a high frequency. The repertoire of LOS seen following SDS-PAGE separation represents the contributions of each of the variants. The relative abundance of each LOS would reflect the relative abundance of the variant that makes it within the total population. The presence of small amounts of the 3.6-kDa LOS of the N variant among the LOSs extracted from populations of R and P variants, and of R and P variant-associated LOS in extracts from N variant populations, would result from the high frequency with which the variants arise. Any mass of variant cells sufficient for extraction of an analyzable amount of LOS could contain many revertants that made additional LOSs unique to them.

In a previous study of phenotypic variation, we also used strain 4505 and MAbs 3F11 and O6B4 (3). Because the two antibodies find their epitopes on LOSs of the same electrophoretic mobility, we were able to analyze variation only in epitope expression and not in production of the LOS molecules themselves.

The regulation of neither LOS production nor the repertoire of their epitopes is understood. The LOS repertoire of a strain can be altered by DNA transformation (19a) or by selection of pyocin-resistant mutants (4, 10). But it can also spontaneously change in the absence of either selective pressure or intentional genetic alterations, as the present data show. Furthermore, both the physicochemical and epitopic expression of LOSs vary with phase of growth (3) and available nutrients (11).

The heterogeneity of neisserial LOS reflects physicochemical differences in their oligosaccharides (5, 21). Heterogeneity in expression of LOS epitopes is also a reflection of oligosaccharide heterogeneity (5, 7; Griffiss et al., in press). There are three primary mechanisms by which heterogeneity could take place. Regulation at the level of gene expression would determine which glycosylating enzymes a cell produces; availability of substrate and the chemical milieu in which the cell resides would affect the efficiency of glyco-

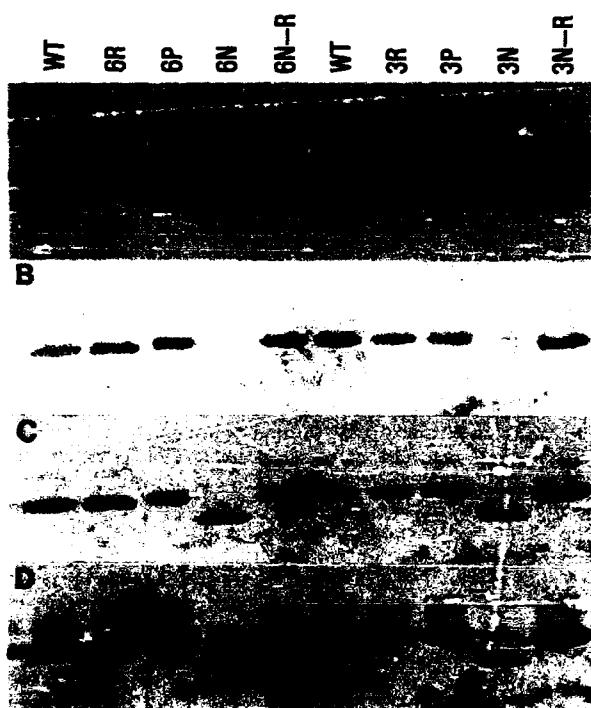


FIG. 3. Comparison of SDS-PAGE profiles and MAb reactivity of the LOS of WT 4505 and its O6B4 and 3F11 MAb-selected variants. Lanes prefixed with 6 indicate variants selected with O6B4 MAb; those prefixed with 3 indicate variants selected with 3F11 MAb. (A) Silver stain of SDS-PAGE-separated LOS of WT 4505 and its MAb-selected variants. The remaining panels are an immunoblot of a replica of gel A treated sequentially as indicated. (B) 3F11 MAb and alkaline phosphatase-conjugated secondary antibody; (C) 2-1-L8 MAb and horseradish peroxidase-conjugated secondary antibody; (D) O6B4 MAb and horseradish peroxidase-conjugated secondary antibody (superimposed on 3F11-reactive LOS components).



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FIG. 4. IEM of O6B4 MAb-selected 4505 R variant organisms. (A) R variant organisms; note that deposition of immuno-colloidal gold particles is distal from the surfaces of the organisms. (B) P variant organisms; deposition of immuno-colloidal gold particles is closer to the cell surface than on R variants. (C) N variant organisms; only rare colloidal gold particles can be seen. Bars, 0.1 μ m.

sylation; and finally, additional interactions between glycosidic moieties and other outer membrane constituents would determine conformation and epitope expression. Combinations of these separate regulatory mechanisms would explain the various data in this and previous studies (3, 7, 8, 17).

The structural basis of the obvious difference in qualitative expression of the 3F11- and O6B4-defined epitopes by the R and P variants remains unclear to us. The circumferential and distal pattern of immuno-colloidal gold deposition around the R variants is curious. It resembles the deposition of ferritin-labeled antibody binding to a capsular structure, as has been described for the binding of antibodies to lipopolysaccharide antigens on enteric bacteria (18). The length of the oligosaccharide of a neisserial LOS is determined by the length of what we have termed the elongation segment, an internal segment in the oligosaccharide consisting of a variable number of hexose residues (13; B. Gibson, J. Webb, S. Fisher, A. Burlingame, R. Yamasaki, and J. M. Griffiss, Abstr. 35th ASMC Conf. Mass Spectrometry, 1987, p. 876-877; Griffiss et al., in press). The terminal trisaccharide or tetrasaccharide segment that contains the epitopes defined by 3F11 and O6B4 (4, 10) would not, therefore, be affected by the length of the oligosaccharide.

It is tempting to reason, by analogy with enteric organisms, that the R variant makes a hyperelongated LOS that terminates in the glycosidic sequence bearing the 3F11- and O6B4-defined epitopes, whereas the same sequence is borne on the shorter oligosaccharides of the P variant LOS. However, the electrophoretic mobility of LOS from the two variants was the same, leaving us with no explanation for our results.

Poolman et al. (14) reported a similar binding pattern of LOS antibody to an *N. meningitidis* variant. They also found that antibodies specific for protein epitopes were excluded from the surface of this meningococcal variant. Whether this will be true for gonococcal variants needs to be explored, for it emphasizes the potential importance of phase variation in one surface structure on biological phenomena that are dependent on a different surface structure.

These studies show that the degree and manner in which LOS epitopes are expressed is regulated at the single-cell level. However, when LOS epitope expression is assessed in terms of a colony or multiple colonies, it is a stable population attribute. The high frequencies with which we detected *in vitro* variation and reversion may signal the constant presence of LOS variants *in vivo* and may be a unique means by which *N. gonorrhoeae* evades the host immunologic defenses.

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